

AWARD NUMBER: W81XWH-07-1-0121

TITLE: Humanized in vivo Model for Autoimmune Diabetes

PRINCIPAL INVESTIGATOR: Gerald T Nepom, M.D., Ph.D.
John A Gebe, Ph.D.

CONTRACTING ORGANIZATION: Benaroya Research Institute
Seattle, WA 98101

REPORT DATE: February 2009

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 1 February 2009		2. REPORT TYPE Annual		3. DATES COVERED 8 Jan 2008 – 7 Jan 2009	
4. TITLE AND SUBTITLE Humanized in vivo Model for Autoimmune Diabetes				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-07-1-0121	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Gerald T Nepom, M.D., Ph.D. John A Gebe, Ph.D. E-Mail: nepom@benaroyaresearch.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Benaroya Research Institute Seattle, WA 98101				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The CD4+ T cell response is critical for cellular autoimmunity in human T1D, but incomplete understanding of issues of specific cell frequency, avidity, function, and correlation with disease status presents major obstacles to improved therapies. This research study entails using humanized mice manifesting type 1 diabetes (T1D)-associated human HLA molecules to address the fate and pathogenicity of high and low avidity T cells reactive to the putative autoantigen glutamic acid decarboxylase 65 (GAD65). By modeling the dominant human anti-GAD65 response in HLA- and TCR-transgenic mice, we proposed to determine whether pathogenic and/or regulatory responses correspond to high or low avidity profiles at different points during disease course. These ongoing studies indicate that the tolerance mechanisms used to prevent self-antigen GAD65 reactive T cells from eliciting autoimmunity in humanized DR4 HLA mice are diverse and that no single mechanism is exclusively used to maintain immune tolerance and prevent diabetes.					
15. SUBJECT TERMS Autoimmunity; type 1 diabetes; humanized mouse model; T cell; GAD65					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	19	19b. TELEPHONE NUMBER (include area code)

**United States Army Medical Research and Materiel Command
Research Technical Report**

Contract Number W81XWH-07-1-0121

Table of Contents

	Page
Introduction	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes	6
Conclusion	6
References	6
Appendices	
Statement of Work, 2006	8
Publication, in press	10

Humanized in vivo Model for Autoimmune Diabetes
Gerald T Nepom, MD, PhD; John A Gebe, PhD
Benaroya Research Institute
Seattle, WA 98101-2795
January 2009

Research Technical Report—REVISED May 2009

INTRODUCTION

This research study uses humanized mice containing type 1 diabetes (T1D)-associated human HLA molecules to address the fate and pathogenicity of T cells reactive to the autoantigen glutamic acid decarboxylase 65 (GAD65).

BODY

The focus in the second year of this grant has been on **Task 1** (Statement of Work, 2006, appended): To evaluate diabetes disease progression in the DR4/TCR models in order to test the hypothesis that high avidity autoreactive CD4⁺ T cells escape from selection and persist in the periphery as dominant clonotypes; and to evaluate the fate and pathogenicity of high and low avidity autoreactive T cells representative of the human T1D repertoire.

In a follow up to last year's report on 164 and 4.13 TcR transgenic mice responsive to naturally processed GAD65 555-567, we now report (enclosed manuscript submitted) that these two T cells, both of which use Va12.1/Vb5.1 structural sequences and differ only in their CDR3 regions, display stark differences in both central and peripheral tolerance mechanisms to maintain peripheral self-tolerance when expressed as TcR transgenes. These differences make them an ideal model for our overall objectives to understand and modify the differing autoreactive T cells in humans.

In addition to the strong negative thymic selection (central tolerance) displayed in 164 mice, peripheral tolerance is also achieved through activation-induced cell death (AICD). Peripheral self-antigen reactive 164 T cells are activated in the periphery in spleen and lymph nodes and are also Annexin V, active Caspase 3, and CD95 positive (see enclosed manuscript, Figure 3). The expression of these molecules specific to 164 T cells, but not 4.13 T cells, is indicative of peripheral tolerance through AICD as a method to maintain tolerance to self antigen^{1,2}. This is likely the primary mechanism for lack of disease progression in these cases, and is sufficient to block disease induction by agonists or Treg deletion (**Task 1b, Task 1e, and Task 1f**).

In contrast, T cells from 4.13 mice are not activated in vivo but, like 164 T cells, do secrete IFN- γ upon in vitro stimulation (see enclosed manuscript, Figure 5). Interestingly, unlike 164 T cells, 4.13 CD4 T cells upon stimulation secrete IL-10 independently of IFN- γ (see enclosed manuscript, Figure 6). The differentiation into IL-10-producing cells (presumably Tr1 type) is a peripheral differentiating event, as proliferating mature T cells from the thymus do not secrete the cytokine (see enclosed manuscript, Figure 7). As IL-

10 has been shown to regulate immune responses^{3,4}, these mice have been crossed onto IL-10-deficient mice to test the hypothesis that: 1. IL-10 is preventing these particular GAD65 self-antigen-responsive 4.13 cells from activation that is in contrast to 164 T cells; and 2. the lack of in vivo activation of 4.13 T cells by the IL-10-secreting cells is preventing their migration into the pancreatic islets, as was observed in 164 mice (**Task 1e**). Spontaneous islet infiltration has also not been observed in 4.13 mice on a Rag2^{o/o} background (**Task 1f**).

We recently reported that 164 mice on a Rag2^{o/o} background exhibited a T cell pancreatic islet infiltrate, which was associated with loss of islet insulin in infiltrated islets. However, the disease process in these mice did not continue to progress to frank diabetes. This intriguing finding leads to three alternatives:

1. The B cell compartment may regulate diabetes induction in our model⁵. HLA transgenic diabetic RIP-B7/DR4 mice do have a B cell islet infiltrate⁶. 164 mice have been crossed onto TcR Ca^{o/o} mice to maintain monoclonality of the T cell repertoire and reintroduce the B cell compartment, and they are currently being monitored for islet infiltrate and diabetes.
2. In both the NOD mouse and human disease, other genetic components contribute to disease⁷. To study IL-2 and its association with Treg function, we are currently crossing 164 mice to B6 congenic strains containing both insulin-dependent diabetes (idd3) (IL-2-containing loci) and idd5-susceptible loci.
3. Recent work has shown the importance of ROR γ t-mediated Th17 cells in both experimental autoimmune encephalomyelitis⁸ and collagen-induced arthritis⁹ models of autoimmunity. Through a collaboration, we have obtained CD4 promotor-driven ROR γ t mice and are crossing them to 164 mice to skew the cytokine profile from a Th1 (IFN- γ secreting) to a Th17 profile.

KEY RESEARCH ACCOMPLISHMENTS

- Tolerance to autoreactive Th1 164 T cells is maintained through a strong thymic negative selection (central tolerance) and also in the periphery by activation-induced cell death (peripheral tolerance).
- Central tolerance in 4.13 mice is also achieved by negative selection, albeit to a lesser extent, likely a reflection of its lower avidity. In contrast to peripherally activated 164 T cells, peripheral 4.13 T cells appear to achieve tolerance through the generation of IL-10-secreting Tr1 cells. This hypothesis is currently being tested by crossing 4.13 mice onto IL-10 knockout mice.
- The generation of potentially regulatory IL-10-secreting, peripheral 4.13 T cells in 4.13 TcR transgenic mice is a post-thymic, peripheral differentiating event, as mature CD4+/CD8- thymocytes do not secrete IL-10 upon autoantigenic stimulation.

- In contrast to 164 mice on a Rag2^{o/o} background, 4.13 mice on a Rag2^{o/o} background do not exhibit islet infiltrates.
- 164 mice have been successfully crossed onto TcR Ca^{o/o} mice. We can now test the hypothesis that the B cell compartment of the immune system will lead to a more severe form of insulinitis and/or diabetes in 164 mice.

REPORTABLE OUTCOMES—Manuscripts

Gebe JA, Yue BB, Unrath KA, Falk BA, and Nepom GT. Restricted autoantigen recognition associated with deletional and adaptive regulatory mechanisms. *J Immunol*, in press, 2009.

CONCLUSION

In a detailed analysis of tolerance in GAD65 555-567-responsive 164 and 4.13 TcR transgenic mice, we find that tolerance in 164 TcR transgenic mice is maintained at both the central level (thymic deletion) but also peripherally through Caspase 3-mediated AICD. This finding contrasts sharply to 4.13 TcR mice where peripheral tolerance through AICD is not evident. Furthermore, we find the differentiation of 4.13 T cells into IL-10-secreting Tr1 cells is a peripheral differentiating event as evident by a lack of IL-10 cytokine in stimulated mature CD4⁺ thymocytes. As both T cells use identical Va12.1/Vb5.1 TcR segments, these data indicate that subtle sequence changes in the CDR3 region of TcRs responsive to the same antigen can lead to drastically different mechanisms of tolerance induction.

While autoreactive 164 TcR mice on a Rag2-deficient background exhibit a pancreatic infiltration¹⁰, we have not observed this to be the case for 4.13 mice. It is tempting to speculate that the IL-10-secreting Tr1 nature of the 4.13 T cells may be responsible for preventing both the peripheral activation and islet infiltration, which is observed in 164 mice. Having generated IL-10 knockout mice containing 4.13 TcR, we are now in a position to answer this question. Having also crossed our TcR mice onto ROR γ t mice, we also can address the influence of the generation of Th17 cells in the islet infiltrative process in seen in 164 mice.

Understanding of these mechanisms that control the autoreactivity of the human 4.13 and 164 TcR will lead to informed choices for selecting and monitoring novel immunotherapy.

REFERENCES

1. Zhang HG, Su X, Liu D, Liu W, Yang P, Wang Z, Edwards CK, Bluethmann H, Mountz JD, Zhou T. Induction of specific T cell tolerance by Fas ligand-expressing antigen-presenting cells. *J Immunol* 1999 February 1;162:1423-30.

2. Herndon JM, Stuart PM, Ferguson TA. Peripheral deletion of antigen-specific T cells leads to long-term tolerance mediated by CD8⁺ cytotoxic cells. *J Immunol* 2005 April 1;174:4098-104.
3. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993 October 22;75:263-74.
4. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997 October 16;389:737-42.
5. Noorchashm H, Noorchashm N, Kern J, Rostami SY, Barker CF, Naji A. B-cells are required for the initiation of insulinitis and sialitis in nonobese diabetic mice. *Diabetes* 1997 June;46:941-6.
6. Gebe JA, Unrath KA, Falk BA, Ito K, Wen L, Daniels TL, Lernmark A, Nepom GT. Age-dependent loss of tolerance to an immunodominant epitope of glutamic acid decarboxylase in diabetic-prone RIP-B7/DR4 mice. *Clin Immunol* 2006 December;121:294-304.
7. Maier LM, Wicker LS. Genetic susceptibility to type 1 diabetes. *Curr Opin Immunol* 2005 December;17:601-8.
8. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 2006 July 1;177:566-73.
9. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J Immunol* 2003 December 1;171:6173-7.
10. Gebe JA, Unrath KA, Yue BB, Miyake T, Falk BA, Nepom GT. Autoreactive human T-cell receptor initiates insulinitis and impaired glucose tolerance in HLA DR4 transgenic mice. *J Autoimmun* 2008 June;30:197-206.

APPENDIX

Gebe JA, Yue BB, Unrath KA, Falk BA, and Nepom GT. Restricted autoantigen recognition associated with deletional and adaptive regulatory mechanisms. *J Immunol*, in press, 2009.

Humanized in vivo Model for Autoimmune Diabetes
Gerald T Nepom, MD, PhD; John A Gebe, PhD
Benaroya Research Institute
Seattle, WA 98101-2795

Statement of Work (May 2006)

- Task 1.* Evaluate diabetes disease progression in the DR4/TCR models (months 1-18)
- a. Cross the DR4/TCR transgenics onto the DR4/RIP-B7 transgenics for an accelerated disease course (months 1-9)
 - b. Inject TLR agonists and/or anti-CD40 agonists for further disease acceleration (months 1-9)
 - c. Inject low-dose streptozotocin to initiate beta cell damage, testing the impact of antigen release in situ (months 1-9)
 - d. Transfer spleen and LN cells from DR4/TCR transgenic donors, activated in vitro, after labeling with CFSE, to track their in vivo course (months 6-18)
 - e. Deplete donor cells of the CD4+CD25+ regulatory population prior to transfer; alternatively, supplement donor cells with enriched CD4+CD25+ cells from avidity-selected TCR transgenic donors (months 6-18)
 - f. In all cases (a-e), monitor immunohistochemistry of pancreatic islets, using staining for CD4, FOXP3, and insulin, as well as standard H&E. In all cases, monitor blood glucose and intraperitoneal glucose tolerance tests at regular intervals (months 1-18)
- Task 2.* Evaluate tetramer profiles and disease progression in the TCR 164b model (months 12-42)
- a. Measure insulinitis and glycemia in the DR4/164b transgenics, while monitoring peripheral blood, LN and spleen cells for DR4-GAD tetramer binding profiles (months 12-42)
 - b. Measure cytokines and TCR alpha chain utilization in the GAD-responsive T cells, sorting for tetramer+ and activation profiles; compare high tetramer binding cells with low avidity cells (months 12-42)

- c. Monitor disease progression comparing tetramer binding with the same parameters as (f) above. Do this in the presence of the disease accelerants selected for activity in Task 1 (months 12-42)
- d. Determine if CD4+CD25+ GAD-specific regulatory T cells derive from the low or the high avidity end of the tetramer-binding spectrum, by flow sorting using tetramers and FOXP3 markers (months 12-42)

Task 3. Optimize the translational potential of the tetramer and disease profiles (months 18-42)

- a. Compare peripheral blood tetramer profiles and T cell phenotypes with simultaneous LN and spleen profiles, at different stages of disease progression (months 18-42)
- b. Elute lymphocytes from infiltrated pancreatic islets and evaluate for tetramer binding and FOXP3 staining, at different stages of disease progression (months 18-42)
- c. Create a standardized multiparameter analysis protocol combining tetramer staining with the most informative T cell markers (determined in the above tasks) that is suitable for analysis of peripheral blood-derived lymphocytes (months 18-42)

AQ:A,B

Restricted Autoantigen Recognition Associated with Deletional and Adaptive Regulatory Mechanisms¹

John A. Gebe,^{2*} Betty B. Yue,^{*} Kelly A. Unrath,[†] Ben A. Falk,^{*} and Gerald T. Nepom[‡]

Autoimmune diabetes (T1D) is characterized by CD4⁺ T cell reactivity to a variety of islet-associated Ags. At-risk individuals, genetically predisposed to T1D, often have similar T cell reactivity, but nevertheless fail to progress to clinically overt disease. To study the immune tolerance and regulatory environment permissive for such autoreactive T cells, we expressed TCR transgenes derived from two autoreactive human T cells, 4.13 and 164, in HLA-DR4 transgenic mice on a C57BL/6-derived "diabetes-resistant" background. Both TCR are responsive to an immunodominant epitope of glutamic acid decarboxylase 65₅₅₅₋₅₆₇, which is identical in sequence between humans and mice, is restricted by HLA-DR4, and is a naturally processed self Ag associated with T1D. Although both TCR use the identical V α and V β genes, differing only in CDR3, we found stark differences in the mechanisms utilized *in vivo* in the maintenance of immune tolerance. A combination of thymic deletion (negative selection), TCR down-regulation, and peripheral activation-induced cell death dominated the phenotype of 164 T cells, which nevertheless still maintain their Ag responsiveness in the periphery. In contrast, 4.13 T cells are much less influenced by central and deletional tolerance mechanisms, and instead display a peripheral immune deviation including differentiation into IL-10-secreting Tr1 cells. These findings indicate a distinct set of regulatory alternatives for autoreactive T cells, even within a single highly restricted HLA-peptide-TCR recognition profile. *The Journal of Immunology*, 2009, 183: 0000–0000.

AQ: C

Central and peripheral mechanisms maintaining T cell tolerance to self Ags are variable in degree of completeness, and autoreactive T cells populate the peripheral immune system. Central tolerance in the thymus is largely governed through the interaction of the TCR with self peptide MHC complexes, in which high avidity T cells are eliminated through apoptosis (1, 3) or potentially differentiated into CD4⁺CD25⁺FOXP3 expressing regulatory T cells (Treg)³ (4, 5). Strategies by which autoreactive T cells may escape central tolerance to self Ags include down modulation of receptor or costimulatory molecules (6) and skewing of CD4/CD8 coreceptor expression (7, 8). These mechanisms are incomplete, however, such that self reactivity by some peripheral T cells is an intrinsic property of normal immunity, perhaps required to enable the immune repertoire to respond to the diverse nature of foreign Ags (9).

Once in the periphery, several additional mechanisms operate as checkpoints to limit T cell activation to self Ags, including functional inactivation or anergy of the T cell (10, 11), activation induced T cell deletion (12–14), generation of suppressive cytokine secreting T cells (Tr1 and Th3) (15, 16), and differentiation of uncommitted T cells into FOXP3 expressing regulatory T cells (17, 18).

While several TCR transgenic mice have been developed to study tolerance to self Ags, the vast majority of studies use either alloreactive T cells or a foreign Ag reactive T cell expressed as a TCR transgene along with the foreign Ag as a second transgene (4, 19, 20). In human type 1 diabetes (T1D), HLA DR4 subjects commonly carry peripheral T cells reactive to a variety of islet associated self Ags, including the immunodominant glutamic acid decarboxylase (GAD)65₅₅₅₋₅₆₇ peptide, a naturally processed epitope of glutamic acid decarboxylase (21–24). Interestingly, recognition of this epitope displays a biased TCR repertoire, with prevalent use of V β 5.1/V α 12.1, although CDR3 regions are variable (22). To study tolerance mechanisms associated with this dominant autoreactive specificity, we introduced transgenic TCR from two human CD4⁺ T cells specific for GAD65₅₅₅₋₅₆₇, which differ only in their CDR3 regions, intercrossed into HLA DR4 transgenic mice. Despite the close structural features of these two autoreactive TCR, stark differences in both central and peripheral tolerance mechanisms were elicited.

Materials and Methods

Mice

DR040 IE mice (DR4) were obtained from Taconic. These C57BL/6 I Ab^{0/0} mice express a human mouse chimeric class II molecule in which the TCR interacting peptide binding domains of mouse IE (domains α_1 and β_1 , exon 2 in both genes) have been replaced with the α_1 and β_1 domains from DRA *0101 and DRB *0401, respectively. Retention of the murine α_2 and β_2 domains allows for the cognate murine CD4 murine MHC interaction (25).

TCR sequences for generation of the two T cell transgenic mice were obtained from human CD4⁺ T cell clones 64 (26) and 4.13 (22). Both human T cells are responsive to the same self Ag GAD65₅₅₅₋₅₆₇ and both use human V α 2/V β 5 T cell receptors. The 64 T cell was cloned from peripheral blood from an HLA DRA *0101/DRB *0401 diabetes at risk individual as previously described (26).

Clone 4.13 was cloned from the peripheral blood of an HLA DRA *0101/DRB *0401 diabetic individual (22). Human mouse chimeric TCR transgenes were constructed by subcloning PCR amplified regions encoding rearranged V α 2 and V β 5 domains from the human clones into pTacass and pTbcass TCR transgenic vectors, respectively (27). TCR transgenic vectors pTacass and pTbcass contain the natural mouse TCR α

Benaoya Research Institute, Seattle, WA 98101. [†]Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331 and [‡]Department of Immunology, University of Washington School of Medicine, Seattle, WA 98195.

Received for publication December 3, 2008. Accepted for publication April 12, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹This research was supported by National Institutes of Health Grant AI050864 and United States Army Medical Research Acquisition Act of 2001 Grant PR064261.

²Addressee correspondence and reprint requests to Dr. John A. Gebe, Department of Diabetes, Benaoya Research Institute, 1201 9th Avenue, Seattle, WA 98101. E-mail: addessgebe@benaoyaesearch.org.

³Abbreviations used in this paper: Treg, regulatory T cell; GAD, glutamic acid decarboxylase; T1D, type 1 diabetes.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/\$20.00

and β promoter/enhancer elements and mouse $C\alpha$ and $C\beta$ constant regions, respectively. DNA injection into C57BL/6J Ab^{o/o} (64 TCR) or F₁ B6/C3H (4/3 TCR) mouse embryos was performed at the University of Washington (Seattle, WA) in the Comparative Medicine Animal Facility. Founder mice containing the human TCR transgenes were then crossed onto DR040 IE mice to generate DR4/64 and DR4/43 mice. Additional crosses were made onto Rag2^{o/o} mice. A founder mouse was also identified that contained only the 164 TCR β transgene. This was also crossed onto DR040 IE mice. The 4/3 TCR transgenic mice generated in F₁ B6/C3H were crossed for nine generations into DR040 IE mice. An animal work was approved by the Benaroya Research Institute Animal Care and Use Committee and animals were housed in the Benaroya Research Institute American Association of Laboratory Animal Care accredited animal facility.

Tissue processing and flow cytometry

Thymus, spleen, and lymph node tissues were processed into single cell suspensions by gently pressing through 0.40 μ m cell strainers (BD Falcon; ref no 352340) using the rubber end of a 10 ml tuberculin syringe in DMEM 0 media (Invitrogen; catalog no 965 092) supplemented with 10% FBS (HyClone), 100 μ g/ml penicillin, 100 U/ml streptomycin, 50 μ M 2-ME, 2 mM glutamine, and 1 mM sodium pyruvate (Invitrogen). Cell suspensions were centrifuged at 200 \times g for 5 min, aspirated, and either resuspended in DMEM 0 media (lymph node and thymus) or 2) RBC were lysed (for spleens) using 1 ml of ACK lysing buffer (28) for 5 min at 37°C at which time 30 ml of media was added and cells spun down (200 \times g), aspirated, and resuspended in DMEM 0 media. The following chromophore-labeled Abs were used in flow cytometric analysis: anti-mouse CD4 (clone RM4.5), CD8 (clone 53.6.7), CD25 (clone PC6), CD62L (Me 4), anti-human active caspase 3 (polyclonal, catalog no 55709), CD44 (IM7), Fc γ (2.4G2), and PE-labeled annexin V (from BD Pharmingen), anti-human V β 5 PE (clone IMMUN 57; Immunotech/Coulters), and V α 2 FITC (clone 6D6; Endogen). FACS samples in media were preselected with Fc γ block for 10 min on ice and then stained with specific Abs on ice for 45 min, washed once, and resuspended in FACS staining buffer (PBS containing 1% FBS, 0.1% NaN₃ de) before being run on a FACSCalibur or LSR II flow cytometer (BD Biosciences). Intracellular staining of cells for Foxp3 was performed using eBioscience kit (FJK 6a Ab) according to the manufacturer's instructions. Intracellular staining for active caspase 3, mouse anti-IFN γ (XMG 2; eBioscience), and anti-IL 0 (clone JESS 6E3; eBioscience) was performed using eBioscience intracellular staining kit (catalog no 88 8823 88; eBioscience).

Proliferation assays

In lymph node or purified CD4⁺ T cell proliferation assays $\times 10^5$ lymph node cells were cultured with 2×10^5 (3000 rad) Cs gamma irradiated splenocytes (final volume 150 μ l). Supernatants for cytokine analysis were taken (50 μ l) at 48 h, and 3 H-thymidine was added at 72 h. Thymidine incorporation was assayed at 96 h using a liquid scintillation counter (Wallac PerkinElmer Life Sciences). Splenocyte responses were measured in the same manner using 5×10^5 splenocytes per well. CD4 and CD8 staining positive cells were obtained using Miteny Biotec beads with purity of 90% or greater or by Ab labeling with CD4 and CD8 and sorting by flow cytometry.

Cytokine analysis

Cytokines IL 2, IL 4, IL 5, TNF α , and IFN γ were assayed using a mouse Th1/Th2 cytokine CBA kit (BD Biosciences; catalog no 55 287). IL 0 was assayed using a BD OptEIA mouse IL 0 ELISA set and mouse TGF β was measured using a human/mouse TGF β ELISA Ready SET Go! kit (BD Biosciences; catalog nos 555252 and 88 7344, respectively). Supernatants from triplicate proliferation wells (50 μ l/well) were combined for cytokine analysis, with 50 μ l used for CBA analysis and 50 μ l for IL 0 ELISA.

Results

Thymic selection of autoreactive T cells

Utilizing TCR from two structurally related DRB1*0401 (DR4) restricted human CD4⁺ T cell clones reactive to the autoantigen GAD65, human TCR transgenic mice were generated to investigate differential modes of T cell tolerance to the naturally processed GAD65₅₅₅₋₅₆₇ autoantigen. The human CD4⁺ T cell clones 164 and 4.13 (obtained from two different subjects) are structurally

Table I Comparison of 164 and 4.13 TCR^a

Clone		CDR3 Region sequence											
		TcRAV											
		(-)											
164	51	A	L	S	E	Glu	Gly	G	G	Ala	Asn	S	K
4.13	51	A	L	S	E	N	R	G	G	A	N	S	K
						Asn	Arg			Thr	Ala		
						(+)							
		TcRBV											
164	121	A	S	S	L	Ala	G	G	A	Asn	Pro	Leu	His
4.13	121	A	S	S	L	V	G	G	P	Asn	Pro	Leu	His
						Val			Pro	Ser	Glu	Ala	Phe
						(-)							

^a Gay highlighted areas denote differences between 164 and 4.13 TCR CD3 sequences: boldface, nonpolar-to-nonpolar amino acid changes; underlined, residues, polar-to-polar changes; and parentheses, charge changes.

related in that they both use TCR with human V α 12.1 (hV α 12.1) and V β 5.1 (hV β 5.1) gene sequences, which differ only in their CDR3 regions (Table I). Both of the human T cells recognize GAD65₅₅₅₋₅₆₇ (22, 26), a region within the naturally processed and presented GAD65₅₅₂₋₅₇₂ epitope (21, 23). The sequence of the DR4 binding minimal stimulating epitope GAD65₅₅₅₋₅₆₇ is identical for GAD65 and GAD67 in both human and mouse and thus serves as a naturally processed self Ag T cell epitope in both species (29). Both 4.13/Rag2^{+/+} and 164/Rag2^{+/+} mice display reduced thymus cellularity (Fig. 1A), with 164 mice exhibiting a profound reduction in CD4⁺CD8⁺ double positive cells (Fig. 1B). The reduction in cellularity and a decrease in double positive cells is indicative of negative selection (20, 30, 31). While positively

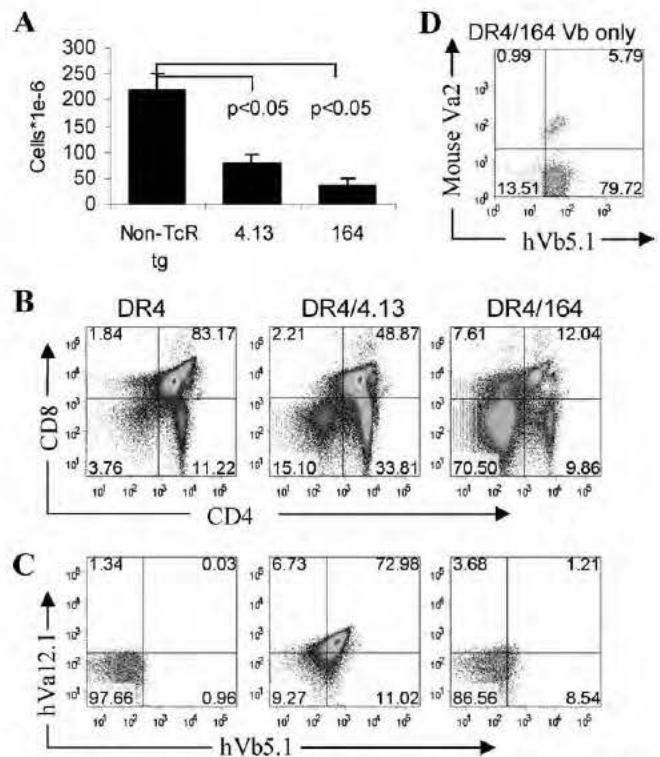
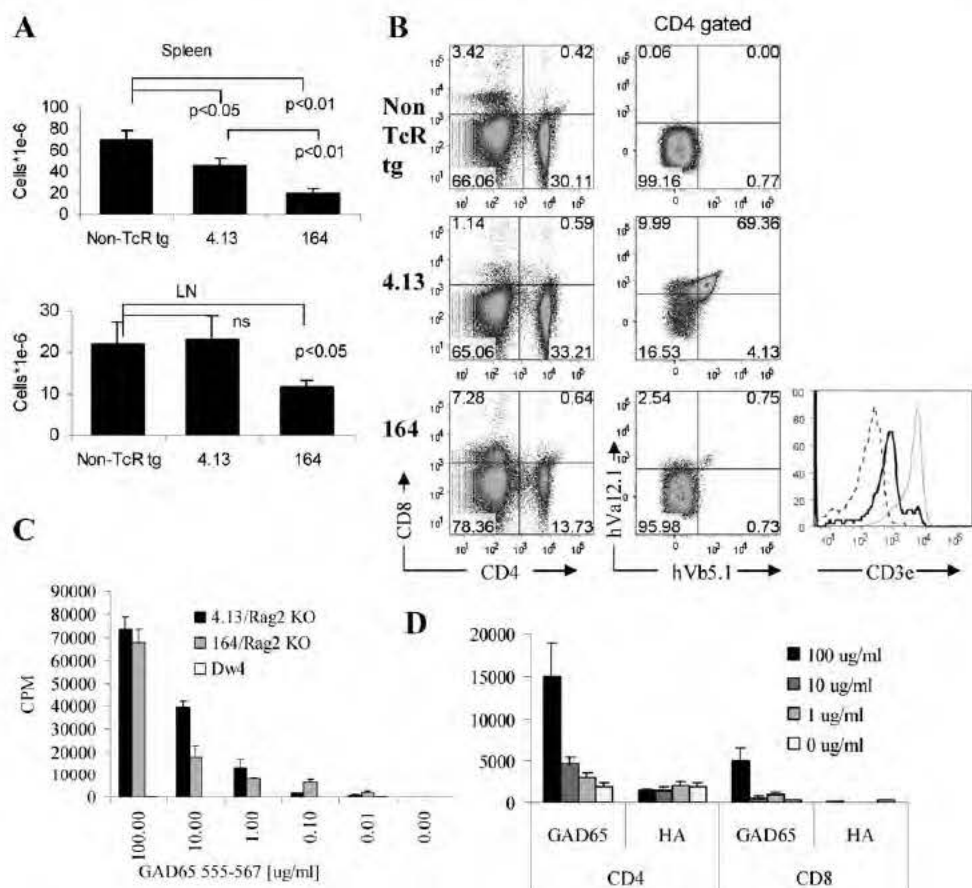


FIGURE 1. Thymic lymphocyte profiles in 164 and 4.13 human TCR transgenic HLA DR4 mice. Thymus cellularity of 8- to 10-week-old mice ($n = 3$) (A), CD4/CD8 profiles (B), and TCR hVb5 vs hVa2 expression on CD4⁺CD8⁻ gated cells (C). Human Vb5 expression on CD4⁺CD8⁻ thymocytes from 64 Vb only TCR mice (D).

FIGURE 2. Peripheral lymphocyte profiles in 8- to 10-wk-old 164 and 4.13 TCR transgenic DR4 mice. Peripheral spleen and lymph nodes (inguinal and para-aortic combined) cellularity ($n = 3$) (A) CD4 vs CD8 profile and TCR human V α 12.1 and V β 5 expression on CD4⁺CD8⁺ gated cells from spleen (B) Histogram in B shows CD3e expression on CD4⁺CD8⁺ gated cells from 164 mice (black line), 4.13 mice (gray line), and isotype control (dotted line). Splenocyte Ag dose response in 164 (gray) and 4.13 (black) TCR transgenic DR4 mice on a Rag2^{o/o} background (C) For CD4⁺CD8⁺ and CD4⁺CD8⁺ T cell stimulation (D) cells were sorted by flow and tested for proliferation in response to GAD65₅₅₅₋₅₆₇ or control Ag. All experiments were repeated at least three times with similar results.



selected 4.13 T cells are heavily skewed toward a single positive CD4⁺CD8⁺ phenotype reflecting their class II restriction, single-positive thymic T cells in 164 mice are matured into both CD4⁺CD8⁺ and CD4⁺CD8⁺ phenotypes, a profile similar to that observed in other self Ag responsive TCR transgenic mice under conditions of strong negative selection (8). In addition to the stronger negative selection observed in 164 mice is the down modulated expression of the TCR on CD4⁺CD8⁺ thymocytes where only $\sim 1\%$ of mature CD4⁺CD8⁺ T cells express both V α and V β transgenes (Fig. 1C). This is in stark contrast to the $>70\%$ expression of hV α 12.1 and hV β 5.1 on CD4⁺CD8⁺ thymocytes from 4.13 mice. As the amino acid sequence in the CDR3 region of 164 TCR is different from 4.13 TCR, it was possible that the low level of hV β 5.1 and hV α 12.1 staining on 164 mice could be the result of differential binding of the Ab itself; however, the hV β 5.1 Ab does stain the 164 TCR from 164 β chain only TCR transgenic mice (lacking the human TCR V α 12.1 transgene), suggesting that the low level of 164 TCR expression on matured CD4⁺CD8⁺ thymocytes is the result of down modulation of the TCR under thymic selection pressures (Fig. 1D). Based on thymic cellularity, CD4 vs CD8 profiles, and TCR expression levels, we conclude that 164 TCR thymocytes, likely due to a higher avidity for peptide MHC of the 164 TCR relative to the 4.13 TCR, undergo stronger central tolerance and maintain a down modulated TCR.

Peripheral skewing of autoreactive T cells

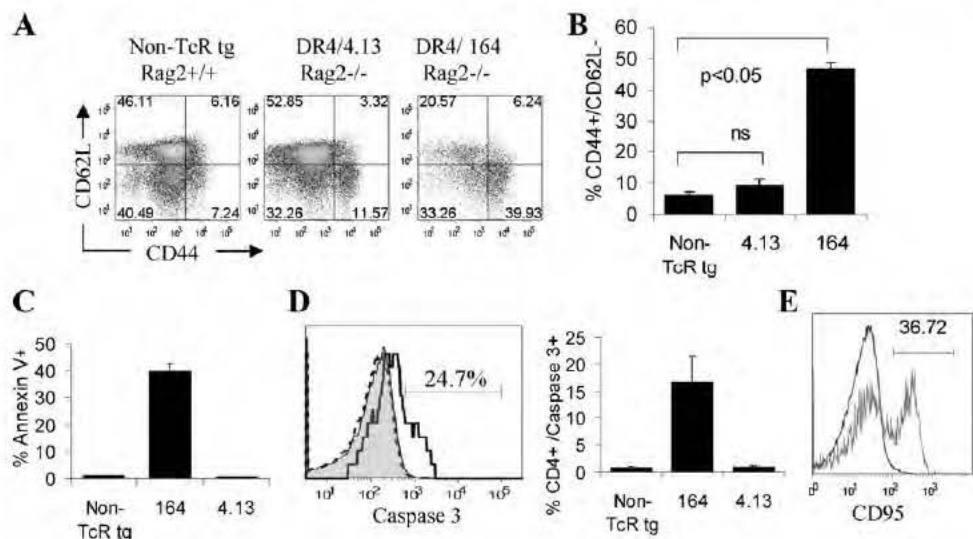
4.13/Rag2^{+/+} and 164/Rag2^{+/+} mice both show reduced cellular density in the spleen (Fig. 2A), but only 164 mice show a reduction in peripheral lymph nodes. The 4.13 T cells in the spleen as in the thymus are heavily skewed toward CD4⁺CD8⁺ lineage, reflecting their class II restriction (Fig. 2B). The 164/Rag2^{+/+} mice have fewer cells in the spleen, and $<1\%$ of CD4⁺CD8⁺ T cells are

hV α 12.1 and hV β 5.1 positive (Fig. 2B). Coinciding with the weak TCR expression in 164/Rag2^{+/+} mice is also a low expression of CD3e on CD4⁺CD8⁺ gated cells (Fig. 2B, histogram). In contrast to the near absence of CD4⁺CD8⁺ cells in 4.13/Rag2^{+/+} mice, 164/Rag2^{+/+} mice have nearly one third of their T cells as CD8⁺CD4⁺ cells, which is also greater than that seen in non TCR transgenic mice (Fig. 2B). The percentages of CD4 cells among all T cells (CD4/(CD4 + CD8)) (average of three mice) are $98 \pm 1\%$ in 4.13 mice and $73 \pm 2\%$ in 164 mice compared with $90 \pm 1\%$ in non TCR transgenic mice, indicating that 4.13 T cells are strongly selected toward their MHC class II restriction, while T cell selection in 164/Rag2^{+/+} mice is skewed toward the CD8 compartment, similar to what is observed in the thymus. The stronger central tolerance in 164/Rag2^{+/+} mice is also reflected in the periphery by the greater expression of endogenous mouse mV α and mV β T cell receptors (supplemental Fig. S1).⁴ In assaying for Ag specificity we used splenocytes from Rag2^{o/o} TCR transgenic mice to ensure that all α/β T cells only express the hV α 12.1 and hV β 5.1 transgenes. Splenocytes from both 4.13/Rag2^{o/o} and 164/Rag2^{o/o} mice respond to GAD65₅₅₅₋₅₆₇ in an Ag-specific manner, confirming their specificity for the GAD65 epitope (Fig. 2C). Because of the skewing of 164 T cells from 164/Rag2^{+/+} mice (also seen in 164/Rag2^{o/o} mice) into a CD8⁺CD4⁺ pathway, we sorted 164/Rag2^{o/o} T cells into CD4⁺CD8⁺ and CD4⁺CD8⁺ fractions and stimulated these fractions with irradiated splenocytes and peptide. We find that both populations are Ag specific, with the CD8 164 cells having a lower proliferative response (lower functional avidity) (Fig. 2D).

Peripheral tolerance mediated by apoptosis

As with the low expression of the transgenic TCR on 164/Rag2^{+/+} thymocytes in the thymus (Fig. 2B), the TCR expression on

FIGURE 3. Activation and apoptosis in 64 and 4.3 TCR transgenic mice. Spleen cells from 8 to 2 wk old DR4 mice on a Rag2^{o/o} background were stained with activation markers CD44 and CD62L and gated on CD4⁺CD8⁻ cells for analysis (A and B). Percentage of gated CD4⁺CD8⁻ cells that were annexin V⁺ (C) and active caspase 3⁺ (D) are shown. Examples of caspase 3 histograms (D) are non TCR transgenic (gray filled), DR4/4.13/Rag2^{o/o} (back dashed line), and DR4/64/Rag2^{o/o} (back heavy line). Percentages are from three mice in each group. CD4⁺ spleen T cells from 64 (gray line) mice are also CD95⁺ compared with non TCR CD4⁺ T cells (back line) (E).



164/Rag2^{+/+} T cells in the periphery is also nearly absent (also true in 164/Rag2^{o/o} mice). This suggested that perhaps the ligand inducing negative selection in the thymus is also activating these cells in the periphery, and thus the extremely low level of TCR expression in the periphery is in part the result of constant activa-

tion of 164 cells in the periphery. By surface phenotyping we found that most peripheral 4.13/Rag2^{o/o} CD4⁺ T cells, like CD4⁺ cells from non TCR/Rag2^{+/+} transgenic mice, are of a naive nature expressing high levels of CD62L and intermediate levels of CD44 (CD62L^{high}CD44^{int}) (Fig. 3A). In contrast, ~40% of peripheral spleen CD4⁺ cells from 164/Rag2^{o/o} mice are CD62L^{low}CD44^{high} compared with ~10% in 4.13 and non TCR transgenic mice, indicating an activated phenotype (Fig. 3, A and B). A similar activation profile of 164/Rag2^{o/o} CD4⁺ T cells was observed in other lymph nodes (pancreatic and inguinal, data not shown) and also in Rag2^{+/+} mice (supplemental Fig. S3). Therefore, we tested whether the low numbers of T cells in the peripheral tissues of 164/Rag2^{o/o} mice could be the result of constant peripheral activation and subsequent activation induced cell death. As shown in Fig. 3C, peripheral CD4⁺ 164/Rag2^{o/o} T cells compared with 4.13/Rag2^{o/o} and non TCR transgenic cells stain with the apoptotic marker annexin V and additional staining indicated that the CD4⁺ 164/Rag2^{o/o} T cells are also activated caspase 3⁺ (Fig. 3D). Peripheral CD4⁺ 4.13/Rag2^{o/o} cells were negative for both annexin V and activated caspase 3 staining. Surface staining on CD4⁺ 164/Rag2^{o/o} T cells indicated that a significant portion of these cells are also CD95⁺, suggesting that apoptotic signaling may occur through CD95 (Fig. 3E).

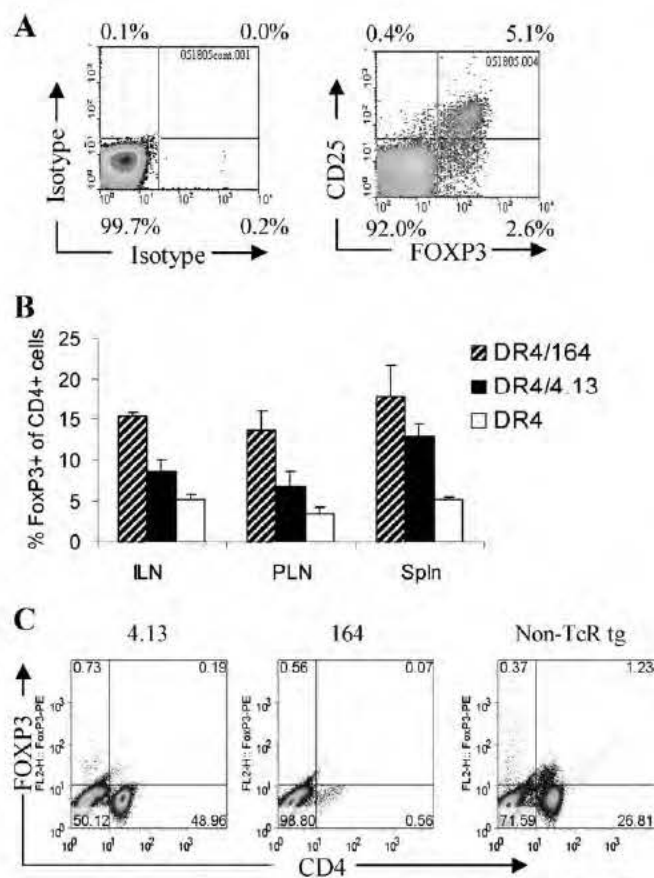


FIGURE 4. Foxp3 expression on spleen CD4⁺CD8⁻ T cells from non TCR transgenic, 64, and 4.3 GAD TCR transgenic mice. Eight to 2 wk old mouse spleen cells from TCR and non TCR transgenic mice were surface stained with CD4, CD25, and then intracellularly for Foxp3. Examples of stainings shown in (A) on non TCR transgenic DR4 spleenocytes. Foxp3 expression in Rag2^{+/+} mice as a percentage of CD4⁺ cells is shown in (B) (average of three mice). Foxp3 expression on CD4⁺ T cells from 64 and 4.3 TCR transgenic mice on a Rag2^{o/o} background is shown in (C).

Both 164 and 4.13 mice show an enhanced selection of peripheral Foxp3⁺ cells

CD4⁺CD25⁺ cells that express Foxp3 participate in immune regulation, and the selection of these Treg can be mediated in foreign

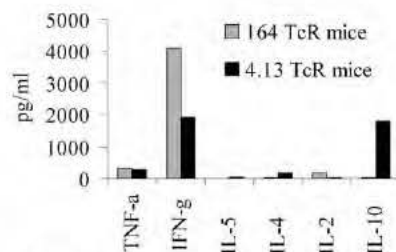
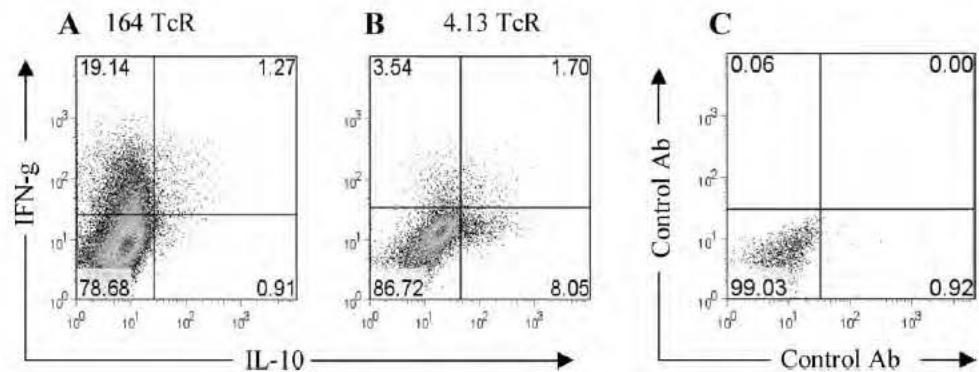


FIGURE 5. Cytokine profile of 164 and 4.13 T cells to GAD65₅₅₂₋₅₇₂. Spleenocytes from DR4/64/Rag2^{o/o} and DR4/4.3/Rag2^{o/o} mice were stimulated with 100 μg/ml GAD65 or control peptide for 96 h. Supernatants were collected at 48 h and TNF-α, IFN-γ, IL-2, IL-4, and IL-5 were measured using a mouse Th1/Th2 kit, and IL-10 was measured by ELISA. Experiment was done three times with similar results.

FIGURE 6. Intracellular staining for IL-10 and IFN- γ in spleen cells from 164 (A) and 4.13 (B) mice were intracellularly stained with IL-10 and IFN- γ directly conjugated Abs. Spleen cells from Rag2^{o/o} 164 and 4.13 mice were stimulated for 4 days with GAD65₅₅₂₋₅₇₂ and then cultured with PMA/onomycin for 4 h with brefeldin A during the last 2 h. Experiment was done three times with similar results.



Ag-specific TCR transgenic mice by expression of the stimulatory Ag as a neo-self peptide driven by tissue-specific promoters (4, 32). It has also been shown that increasing avidity of the TCR for the peptide MHC correlates with a propensity to develop along the thymic derived Foxp3 Treg pathway (4). In our setting involving endogenous self Ag recognition, we find that peripheral CD4⁺ T cells from both autoreactive 4.13/Rag2^{+/+} and 164/Rag2^{+/+} TCR transgenic mice express increased numbers of Foxp3 cells, and that the percentage of CD4⁺ cells that express Foxp3 is highest in 164 mice compared with 4.13 mice, and both are greater than that seen in non TCR transgenic mice (Fig. 4B). However, upon crossing TCR transgenic mice onto a Rag2-deficient background, peripheral Foxp3⁺ cells were near undetectable levels in either 164 or 4.13 mice (Fig. 4C), consistent with the induction of Treg populations in the nontransgenic fraction of endogenous T cells.

Peripheral 4.13 CD4⁺ T cells exhibit Th1 and Tr1 profiles

Cytokine analysis on in vitro stimulated cells from both Rag2^{o/o} TCR transgenic mice responding to GAD65₅₅₅₋₅₆₇ stimulation is shown in Fig. 5. Peripheral 164 T cells are of a Th1 phenotype expressing IFN- γ and little or no IL-4, IL-5, IL-10, or TNF- α , while CD4⁺ 4.13 T cells secrete IFN- γ and IL-10 and little or no IL-4, IL-5, or TNF- α . The same pattern was observed in Rag2^{+/+} mice (data not shown). Because of the unexpected finding of both IFN- γ and IL-10 from GAD65₅₅₅₋₅₆₇ stimulation, we performed

intracellular staining for IFN- γ and IL-10 to determine whether both of these cytokines are derived from the same cell. As shown in Fig. 6, we found that T cells from 4.13/Rag2^{o/o} mice generate IFN- γ independently of IL-10 and therefore peripheral 4.13 CD4⁺ T cells are of a mix of Th1 and Tr1 cells types, while 164 T cells are of a Th1 phenotype generating only IFN- γ . Additional cytokine measurements revealed that 4.13 T cells do not secrete TGF- β 1 (supplemental Fig. S2). Because IL-10 can be immunoregulatory, we addressed whether the commitment of 4.13 T cells to a Tr1 phenotype is a central or peripheral tolerizing event. CD4⁺ T cells from thymus and spleens of DR4/164/Rag2^{o/o} and DR4/4.13/Rag2^{o/o} mice were FACS sorted and stimulated with irradiated APC, and then assayed for IL-10 and IFN- γ production. The 4.13 CD4⁺ T cells from spleen generated IL-10 and IFN- γ in response to either CD3/CD28 or GAD65₅₅₂₋₅₇₂ stimulation, while thymus derived CD4⁺CD8⁻ 4.13 T cells secreted neither cytokine (Fig. 7).

Discussion

Limiting pathogenic autoreactivity is of the utmost importance for a successful immune system, and several mechanisms provide functional checkpoints for this control. These mechanisms broadly fit into three categories: those that involve deletion of autoreactive cells, centrally and/or peripherally; those that involve down modulation of activation molecules or receptors, changing activation thresholds; and those that involve active immune regulation. In this study we evaluated central and peripheral tolerance mechanisms using two TCR transgenic mice containing structurally similar receptors specific for a naturally processed self Ag. These TCR were derived from autoreactive CD4⁺ T cells present in humans with immunity to GAD65, an important islet Ag associated with autoimmune diabetes. On a C57BL/6 "diabetes resistant" background transgenic for HLA DR4, the human class II restricting element for these TCR, very potent in vivo tolerance mechanisms were observed. The 164 TCR was associated with strong deletional events, both in the thymus and in the periphery, and surviving 164 T cells down modulated TCR expression and/or switched from CD4 to CD8 phenotype, even as they maintained specific Ag reactivity. In marked contrast, the 4.13 TCR had less sensitivity to negative selection and no CD4 to CD8 skewing, but instead used a predominant pathway of immunomodulation, skewing toward an IL-10 phenotype.

Both 164 and 4.13 T cells use V α 12.1/V β 5.1 TCR and differ only in CDR3, a region that conventionally interacts primarily with the peptide in the Ag binding MHC (33). Based on the higher thymic cellularity in 4.13 mice compared with 164 mice and the absence of differentiation toward the CD4⁻CD8⁺ pathway, it appears that the 164 TCR is of a higher avidity to peptide MHC complexes in the thymus. As T cell CD4 avidity interaction with

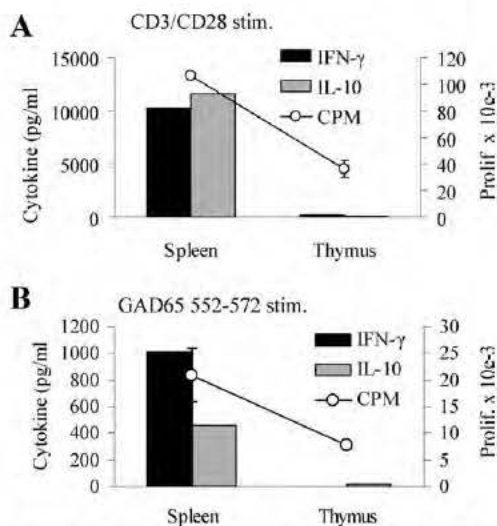


FIGURE 7. IFN- γ and IL-10 production from stimulated sorted CD4⁺CD8⁻ cells from DR4/4.13/Rag2^{+/+} mice. CD4⁺CD8⁻ cells from spleen, lymph node, and thymus were FACS sorted from tissues taken from 8 to 12 wk old mice and stimulated with irradiated APC and either CD3/CD28 Ab (A) or GAD65₅₅₂₋₅₇₂ (B).

the β_2 domain of the MHC class II has been shown to contribute positively to thymic T cell selection (21, 34), the differentiation of immature $CD4^+CD8^+$ double positive 164 thymocytes into $CD4^+CD8^+$ mature cells would presumably lower the TCR over all avidity to the MHC complex and enable escape from negative selection. This skewing toward a $CD4^+CD8^+$ expression pathway and away from a $CD4^+CD8^-$ pathway occurred despite the class II restriction of the original human 164 T cell clone. Consistent with this interpretation is our observation that peripheral $CD4^+CD8^+$ 164/Rag2^{o/o} T cells have less functional avidity to GAD65₅₅₅₋₅₆₇ stimulation than do $CD4^+CD8^-$ 164/Rag2^{o/o} T cells. The skewing of class II restricted self Ag reactive T cells toward a CD8 lineage has been observed in other TCR transgenic models, also in the context of strong negative selection (7, 8).

In addition to thymic deletion and CD4 to CD8 skewing, T cells surviving in the 164 TCR mice showed significant down-regulation of the TCR molecule itself. This also is consistent with a strategy invoked for lowering avidity, and correlated in the mice with evidence of a very strong activation induced cell death pathway. The end result of all these simultaneous high avidity tolerance checkpoints was the presence in the peripheral circulation of a low number of autoreactive T cells, which nevertheless displayed strong Ag-specific proliferative and Th1 characteristics.

Considering that both 164 and 4.13 TCR use $V\alpha 12.1$ and $V\beta 5.1$ and are responsive to the same Ag, it was remarkable that 4.13 T cells showed a completely different tolerance induction profile. A more modest central tolerance for 4.13 T cells was reflected in less thymic deletion and normal $CD4^+CD8^-$ maturation, and similarly no evidence for peripheral activation induced cell death or receptor down modulation was observed. A likely explanation for the absence of peripheral activation of 4.13 T cells was the peripheral generation of IL 10 producing Tr1 regulatory cells in these mice. IL 10 is a potent regulatory cytokine and has been shown to be important in regulating colitis and autoimmunity in experimental autoimmune encephalomyelitis and collagen induced arthritis models (35–38). The absence of IL 10 from sorted $CD4^+CD8^-$ T cells from the thymus upon stimulation with either CD3/CD28 or Ag-specific GAD65₅₅₂₋₅₇₂ peptide also indicates that generation of these IL 10 secreting T cells was a peripheral differentiation event. It is interesting to speculate that T cell generated IL 10 in 4.13 mice could be preventing the activation of 4.13 T cells in the periphery, which contrasts with the activated phenotype in peripheral 164 mice. This hypothesis is currently being testing by crossing DR4/4.13/Rag2^{o/o} mice onto IL-10-deficient mice. While both 164 and 4.13 peripheral T cells are specific for GAD65₅₅₅₋₅₆₇, because many TCR are degenerate in peptide recognition (39), we cannot exclude the possibility that cross reactivity with other unknown ligands might contribute to the differences in functional profiles.

In the periphery, both 4.13 and 164 mice show an increase in Foxp3⁺ cells, which is consistent with that seen in quasi self Ag models (4, 40). The larger increase in the percentage of Foxp3⁺ cells in 164 mice relative to 4.13 mice correlates with the increase in negative selection (higher avidity TCR) in the thymus. However, upon crossing to Rag2-deficient mice we did not detect peripheral $CD4^+CD25^+$ (Foxp3⁺) cells from either 164 or 4.13 mice. This is in contrast to HA-specific and OVA-specific TCR transgenic mice on a Rag-deficient background where the Ag is expressed as a neo self Ag (40–42). In these models up to half of peripheral T cells are CD25⁺ and have a regulatory function. However, in TCR transgenic mice where the T cell responsive Ag is endogenously expressed, $CD4^+CD25^+$ (Foxp3⁺) Treg do not develop on a Rag-deficient background. This includes a myelin basic protein-specific TCR (43) and the BDC2.5 TCR (44). It has

been suggested that a high avidity interaction between T cells and APC in the thymus is required for Treg development (45). Considering the strong negative selection in the thymus of both TCR mice suggesting a high functional avidity of the TCR for MHC Ag, we were surprised to not find $CD4^+CD25^+$ Foxp3⁺ cells in the periphery on Rag2^{o/o} mice. A possible explanation for a lack of Foxp3⁺ Treg in these mice may be that both of these TCR are of high enough avidity that they are beyond the threshold for Foxp3 differentiation (5).

Peripheral tolerance methods of anergy (10, 11), deletion (12–14), or the generation of Tr1(15) and Th3(16) cells are a second line of defense against T cell autoimmunity. Once in the periphery 164 cells displayed a strong activation phenotype in both spleen and lymph nodes resulting in continued down modulation of their TCR and concomitant activation induced cell death through an activated caspase 3 pathway. Consistent with this is the expression of CD95 (FAS) on 164 T cells through which signaling has been shown to mediate deletion induced peripheral tolerance (46, 47). The 4.13 T cells, which populate the periphery to a greater extent, do not undergo this type of peripheral tolerance, most likely due to their apparent lower overall pMHC avidity.

Autoreactive cells, such as those used to derive the 164 and 4.13 TCR in this study, occur frequently in humans with autoimmune disease, in people who are genetically at risk of autoimmune disease, and in normal HLA matched individuals (48–52). Nevertheless, overt autoimmune disease is relatively rare, reflecting the importance of tolerance checkpoints in normal immune function. Our study, using human autoimmune TCR and human MHC transgenic mice, directly demonstrates multiple mechanisms that, sometimes simultaneously, elicit both central and peripheral tolerance. In deed, the two structurally similar TCR used, derived from human HLA-DR4 subjects, with specificity for the same Ag and restriction element and differing only in their CDR3 regions, revealed stark differences in deletional, compensatory, and immunomodulatory mechanisms. That such distinction occurs even with closely related autoreactive TCR underscores the importance of understanding the contribution of this variation to disease susceptibility, pathogenic pathways, and response to therapy.

Acknowledgments

We acknowledge Ace Long for critical review of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References

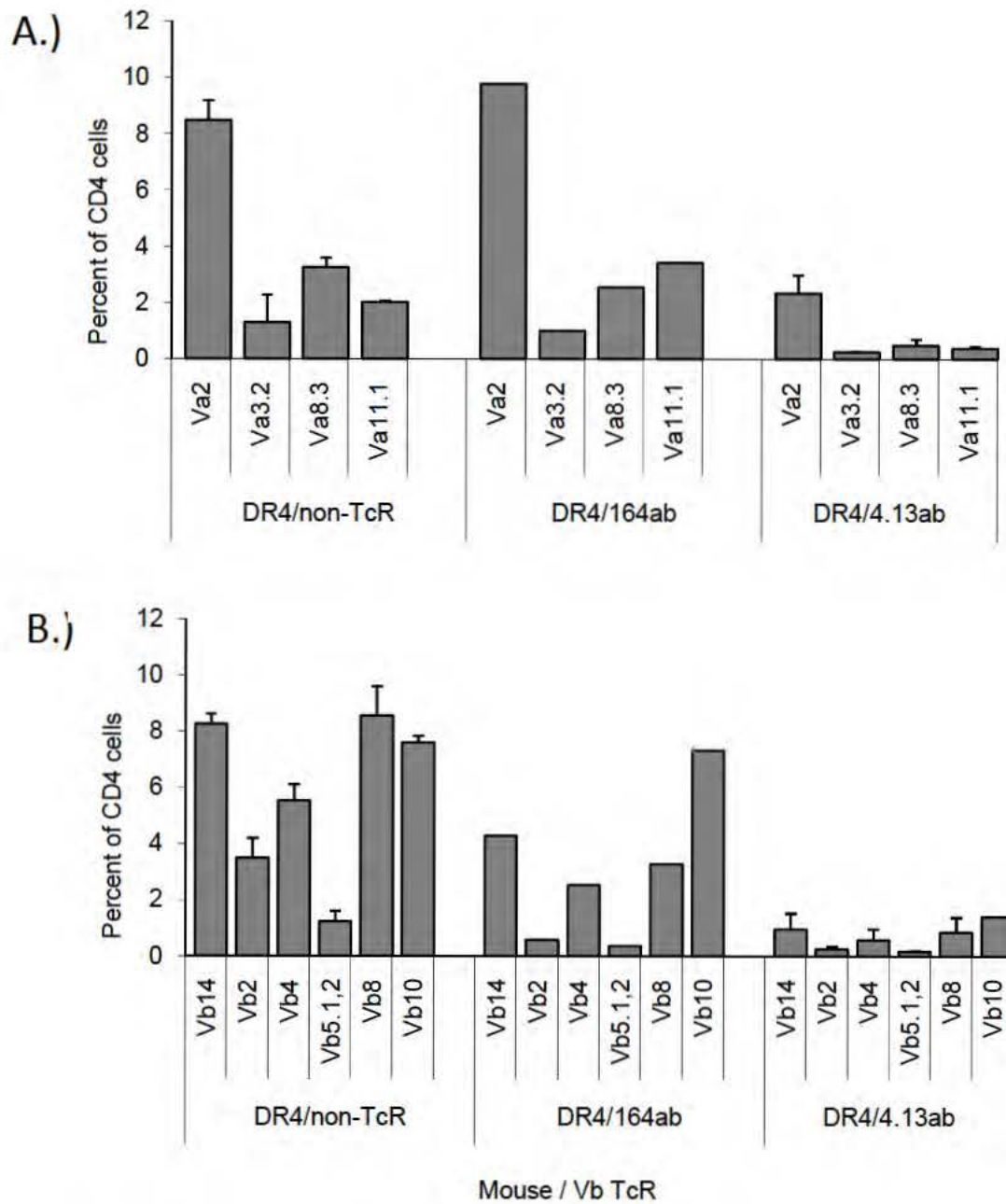
- Bevan, M. J., K. A. Hogquist, and S. C. Jameson. 1994. Selecting the T cell receptor to the *Science* 264: 796–797.
- Lo, D., C. R. Relly, L. C. Buckley, J. DeKoning, T. M. Laufer, and L. H. Glimcher. 1997. Thymic stromal cell specialization and the T-cell receptor to the *Immunol. Res.* 16: 3–14.
- Jameson, S. C., and M. J. Bevan. 1998. T-cell selection. *Curr. Opin. Immunol.* 10: 214–219.
- Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Hohenbeck, M. A. Leiman, A. Na, and A. J. Caton. 2001. Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2: 301–306.
- Feher, Z., and S. Sakaguchi. 2004. $CD4^+$ Tregs and immune control. *J. Clin. Invest.* 114: 1209–1217.
- Banden, M. J., W. R. Heath, and F. R. Calzone. 1997. Down-modulation of CD8 β -chain response to an altered peptide ligand enables developing thymocytes to escape negative selection. *Cell. Immunol.* 175: 111–119.
- Badam, E., L. Ma, and S. Qu. 2005. High incidence of spontaneous autoimmune thyroiditis in immunocompetent self-reactive human T cell receptor transgenic mice. *J. Autoimmun.* 24: 85–91.
- Ranheim, E. A., K. V. Tabbell, M. K. Ogsgaard, V. Mallet-Desgnee, L. Teyton, H. O. McDevitt, and I. L. Weissman. 2004. Selection of abeyant class II restricted $CD8^+$ T cells in NOD mice expressing a glutamic acid decarboxylase (GAD)65-specific T cell receptor transgene. *Autoimmunity* 37: 555–567.
- Goodnow, C. C. 1996. Balancing immunity and tolerance: deleting and tuning lymphocyte responses. *Proc. Natl. Acad. Sci. USA* 93: 2264–2271.
- Schwartz, R. H. 2003. T cell anergy. *Annu. Rev. Immunol.* 21: 305–334.

AQ: F

AQ: G

— AQ: H

- 11 Sabl, S. D., E. K. Deenck, and P. S. Ohashi. 2007. The sound of silence: modulating antigen presentation to T lymphocytes. *Curr. Opin. Immunol.* 19: 658–664.
- 12 Redmond, W. L., C. H. Wei, H. T. K. Euwel, and L. A. Sherman. 2008. The apoptotic pathway controls the deletion of naive CD8⁺ T cells during the induction of peripheral tolerance to a cross-presented self-antigen. *J. Immunol.* 180: 5275–5282.
- 13 Foster, I., and I. L. Ebner. 1996. Peripheral tolerance of CD4⁺ T cells follows local activation in adolescent mice. *Eur. J. Immunol.* 26: 3194–3202.
- 14 Morgan, D. J., H. T. K. Euwel, and L. A. Sherman. 1999. Antigen concentration and peptide frequency determine the fate of CD8⁺ T cell tolerance to peripherally expressed antigens. *J. Immunol.* 163: 723–727.
- 15 You, S., C. Chen, W. H. Lee, T. B. Usko, M. Atkinson, and C. P. Lu. 2004. Presence of diabetes-inhibiting, glutamic acid decarboxylase-specific, IL-10-dependent, regulatory T cells in naive nonobese diabetic mice. *J. Immunol.* 173: 6777–6785.
- 16 Chen, Y., V. K. Kuchroo, J. Inobe, D. A. Hafler, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance suppress auto immune encephalomyelitis. *Science* 265: 1237–1240.
- 17 Cuotode Laffalle, M. A., N. Kutchukhaze, S. Shen, Y. Deng, H. Yee, and J. J. Laffalle. 2008. Adaptive Foxp3⁺ regulatory T cell-dependent and independent control of allergic inflammation. *Immunity* 29: 114–126.
- 18 Walke, M. R., B. D. Carson, G. T. Nepom, S. F. Ziegler, and J. H. Buckner. 2005. De novo generation of antigen-specific CD4⁺CD25⁺ regulatory T cells from human CD4⁺. *Proc. Natl. Acad. Sci. USA* 102: 4103–4108.
- 19 Cabañas, J., C. Cassan, F. Magnusson, E. Paggio, L. Ma, S. J. Debnick, B. Kyewski, D. A. Goss, B. L. Salomon, K. Khaza, et al. 2006. Foxp3⁺CD25⁺ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. *Proc. Natl. Acad. Sci. USA* 103: 8453–8458.
- 20 Piche, H., K. Buks, R. Lang, H. Hengartner, and R. M. Zinkernagel. 1989. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature* 342: 559–561.
- 21 Patel, S. D., A. P. Cope, M. Cong, A. T. T. Chen, E. K. M., L. Fugge, D. Whetzel, and G. Sonderstrup-McDevitt. 1997. Identification of immunodominant T cell epitopes of human glutamic acid decarboxylase 65 by using HLA-DR ($\alpha 1$ 0101, $\beta 1$ 0401) transgenic mice. *Proc. Natl. Acad. Sci. USA* 94: 8082–8087.
- 22 Renon, H., R. Mallone, A.-K. Henning, E. M. Laughlin, S. A. Koch, B. Falk, W. W. Kwok, C. Greenbaum, and G. T. Nepom. 2004. GAD65-specific CD4⁺ T-cells with high antigen avidity are a separate peripheral blood of patients with type 1 diabetes. *Diabetes* 53: 1987–1994.
- 23 Nepom, G. T., J. D. Lippolis, F. M. White, S. Masewicz, J. A. Ma, A. Heiman, C. J. Luckey, B. Falk, J. Shabanowitz, D. F. Hunt, et al. 2001. Identification and modulation of a naturally processed T cell epitope from the diabetes-associated autoantigen human glutamic acid decarboxylase 65 (hGAD65). *Proc. Natl. Acad. Sci. USA* 98: 1763–1768.
- 24 Masewicz, S. A., N. Meldrum, V. Gesuk, L. Gau, W. Hagopian, L. Mota, and G. T. Nepom. 2001. Complexity of human immune response profiles for CD4⁺ T cell epitopes from the diabetes autoantigen GAD65. *Autoimmunity* 34: 231–240.
- 25 Ito, K., H. J. Ban, M. Molina, J. Han, J. Magan, E. Saa, C. Belunis, D. R. Bolin, R. A. Ceo, R. Campbell, et al. 1996. HLA-DR4-IE class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. *J. Exp. Med.* 183: 2635–2644.
- 26 Renon, H., E. J. Novak, S. Koch, A. Henning, A. Lu, W. Kwok, and G. T. Nepom. 2002. Detection of GAD65 specific T-cells by MHC class II multimers in type 1 diabetes patients and at-risk subjects. *Diabetes* 51: 1375–1382.
- 27 Kouskoff, V., K. S. Gnoell, C. Benoist, and D. Mathis. 1995. Cassette vector system for expression of T cell receptor genes in transgenic mice. *J. Immunol. Methods* 180: 273–280.
- 28 Kusbek, A. M. 2000. Isolation and functional analysis of mononuclear cell populations. In *Current Protocols in Immunology*, suppl. 39th ed. J. E. Coligan, A. M. Kusbek, D. H. Margulies, E. M. Shevach, and W. Stobo, eds. Wiley, New York, pp. 311–315.
- 29 Gebe, J. A., B. A. Falk, K. A. Rock, S. A. Koch, A. K. Henning, H. Renon, W. W. Kwok, and G. T. Nepom. 2003. Low-avidity recognition by CD4⁺ T cells directed to self-antigens. *Eur. J. Immunol.* 33: 1409–1417.
- 30 Kselow, P., H. Bluthmann, U. D. Staezel, M. Steinmetz, and H. von Boehme. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4⁺ thymocytes. *Nature* 333: 742–746.
- 31 Gallegos, A. M., and M. J. Bevan. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* 200: 1039–1049.
- 32 Kawahata, K., Y. Masaki, M. Yamauchi, S. Tsunekawa, K. Setoguchi, J. Miyazaki, and K. Yamamoto. 2002. Gene expression of CD4⁺CD25⁺ regulatory T cells from auto-reactive T cells simultaneously with the negative selection in the thymus and from nonauto-reactive T cells by endogenous TCR expression. *J. Immunol.* 168: 4399–4405.
- 33 Rudolph, M. G., and I. A. Wilson. 2002. The specificity of TCR/pMHC interaction. *Curr. Opin. Immunol.* 14: 52–65.
- 34 Rbedy, J. M., E. Mostaghel, and C. Doyle. 1998. Disruption of the CD4-mouse heterocompatibility complex class II interaction blocks the development of CD4⁺ T cells in vivo. *Proc. Natl. Acad. Sci. USA* 95: 4493–4498.
- 35 Kuhn, R., J. Lohle, D. Rennick, K. Raweisky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.
- 36 Goux, H., A. O. G. A., M. B. Gle, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncillo. 1997. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
- 37 Bettel, E., M. P. Das, E. D. Howard, H. L. Weiner, R. A. Sobel, and V. K. Kuchroo. 1998. IL-10 secretion in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J. Immunol.* 161: 3299–3306.
- 38 Johansson, A. C., A. S. Hansson, K. S. Nandakumar, J. Backlund, and R. Holmdahl. 2001. IL-10-deficient B10.Q mice develop more severe collagen-induced arthritis, but are protected from arthritis induced with anti-type II collagen antibodies. *J. Immunol.* 167: 3505–3512.
- 39 Mazza, C., and B. Malissen. 2007. What guides MHC-restricted TCR recognition? *Semin. Immunol.* 19: 225–235.
- 40 Walke, L. S., A. Chodos, M. Eggena, H. Dooms, and A. K. Abbas. 2003. Antigen-dependent proliferation of CD4⁺CD25⁺ regulatory T cells in vivo. *J. Exp. Med.* 198: 249–258.
- 41 Leiman, M. A., J. Laik, III, C. Cozzo, M. S. Jordan, and A. J. Caton. 2004. CD4⁺CD25⁺ regulatory T cell epitope formation in response to varying expression of a neo-self-antigen. *J. Immunol.* 173: 236–244.
- 42 Apostolou, I., A. Sakukhan, L. Klein, and H. von Boehme. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 3: 756–763.
- 43 Hori, S., M. Hauray, A. Coutinho, and J. Demengeot. 2002. Specificity requirements for selection and effector functions of CD25⁺ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* 99: 8213–8218.
- 44 Chen, Z., A. E. Heiman, M. Matos, D. Mathis, and C. Benoist. 2005. Where CD4⁺CD25⁺ Treg cells migrate on autoimmune diabetes. *J. Exp. Med.* 202: 1387–1397.
- 45 Walke, L. S. 2004. CD4⁺CD25⁺ Treg development and role? *Immunology* 111: 129–137.
- 46 Zhang, H. G., X. Su, D. Lu, W. Lu, P. Yang, Z. Wang, C. K. Edwards, H. Bluethmann, J. D. Mountz, and T. Zhou. 1999. Induction of specific T cell tolerance by Fas ligand-expressing antigen-presenting cells. *J. Immunol.* 162: 1423–1430.
- 47 Hendon, J. M., P. M. Stutz, and T. A. Ferguson. 2005. Peripheral deletion of antigen-specific T cells leads to long-term tolerance mediated by CD8⁺ cytotoxic cells. *J. Immunol.* 174: 4098–4104.
- 48 Danke, N. A., D. M. Koelle, C. Yee, S. Beheay, and W. W. Kwok. 2004. Auto-reactive T cells in healthy individuals. *J. Immunol.* 172: 5967–5972.
- 49 Oling, V., J. Mattila, J. Ilonen, W. W. Kwok, G. Nepom, M. Knip, O. S. Mell, and H. Reijonen. 2005. GAD65- and proinsulin-specific CD4⁺ T-cells detected by MHC class II tetamers in peripheral blood of type 1 diabetes patients and at-risk subjects. *J. Autoimmun.* 25: 235–243.
- 50 Bethelot, L., D. A. Laplaud, S. Pette, C. Ballet, L. M. Chel, S. Hillon, C. B. Audeaud, F. Connan, F. Lefevre, S. W. Edwards, et al. 2008. Blood CD8⁺ T cell responses against myelin determinants in multiple sclerosis and healthy individuals. *Eur. J. Immunol.* 38: 1889–1899.
- 51 Danke, N. A., J. Yang, C. Greenbaum, and W. W. Kwok. 2005. Comparative study of GAD65-specific CD4⁺ T cells in healthy and type 1 diabetic subjects. *J. Autoimmun.* 25: 303–311.
- 52 Veldman, C. M., K. L. Gebhard, W. Ute, R. Wassmuth, J. G. Oetgen, E. Schultz, and M. Heitl. 2004. T cell recognition of desmoglein 3 peptides in patients with pemphigus vulgaris and healthy individuals. *J. Immunol.* 172: 3883–3892.



S1. TcR Valpha (A) and Vbeta (B) expression on CD4 gated T cells in DR4 non-TcR tg, DR4/164ab, and DR4/4.13ab mice. Data are from mice between the ages of 8-12 weeks

Supplemental Figure S2

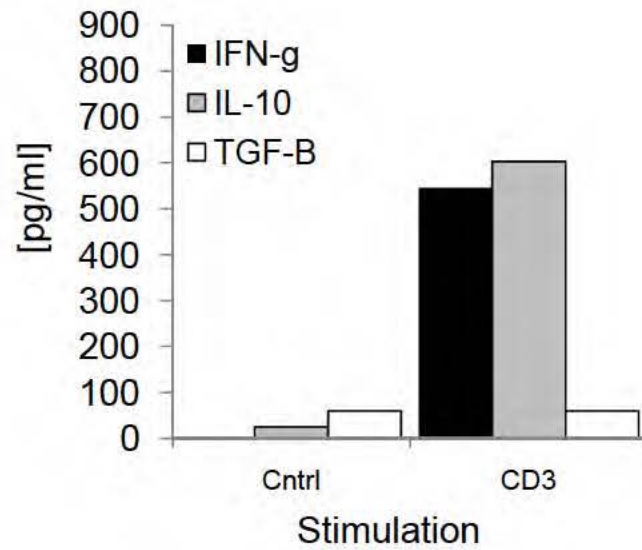


Figure S2. 4.13 TcR transgenic mice secrete IFN-g and IL-10, but not TGF-b1 upon stimulation. Purified CD4⁺ cells from DR4/4.13 mice were stimulated with anti-CD3/CD28 at 2.0/0.2 ug/ml. Supernatants were taken at 72 hours and assayed for cytokines. Limit of detection for TGF-b1 was 60 pg/ml

Supplemental Figure S3

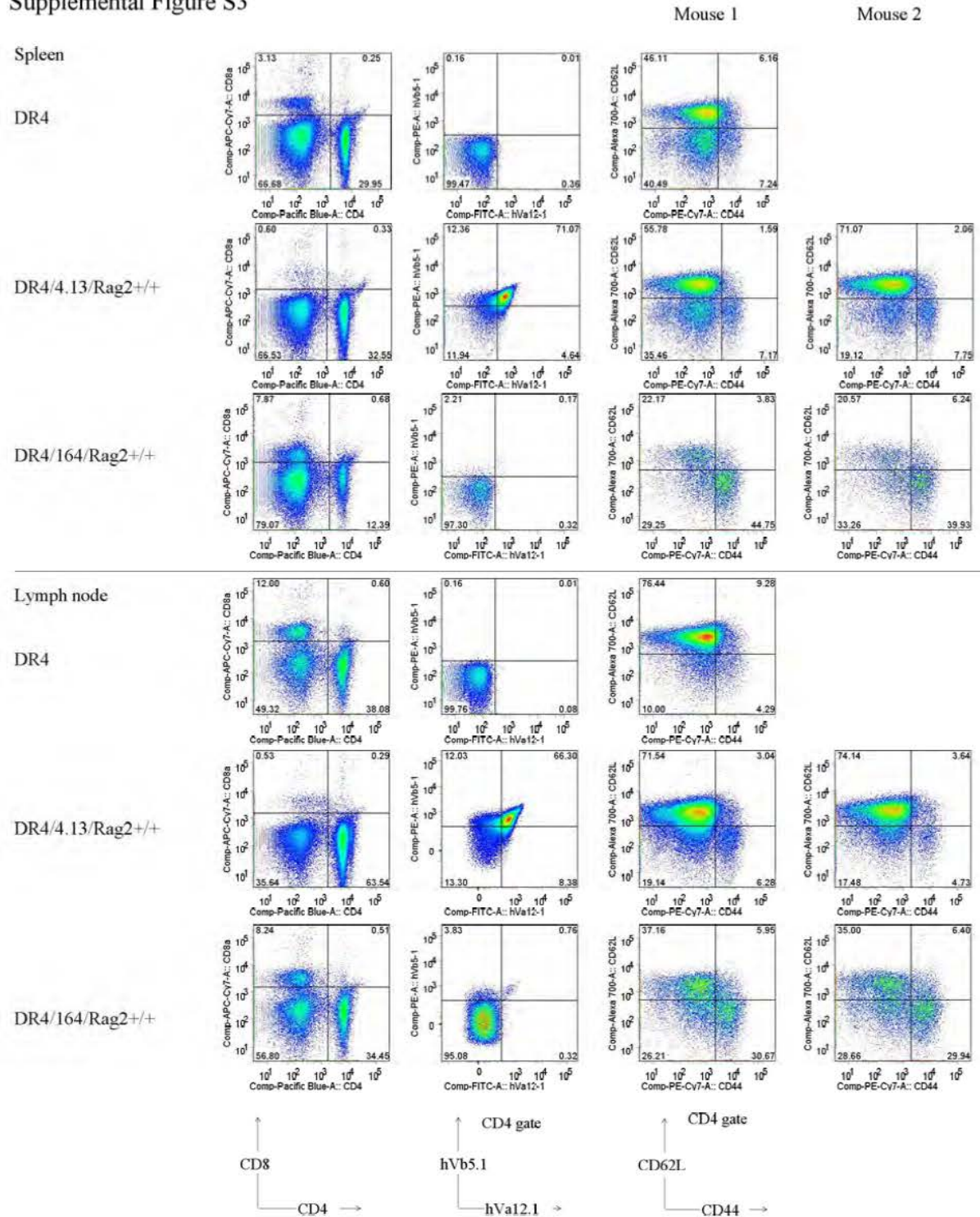


Figure S3. Surface phenotyping of spleen and lymph node cells from non-TcR transgenic 164/Rag2^{+/+}, and 4.13/Rag2^{+/+} transgenic DR4 mice. Human TcR staining and CD44 vs CD62L Expression were done on CD4⁺/CD8⁻ gated cells. Mice were 8-12 weeks of age.